REMARKS

1. Disposition of Claims

Claims 1-3, 5 and 6, and 9-13 are pending in this application. Claims 4, 8, and 14-22 have been canceled as being drawn to non-elected subject matter. No new matter has been added. Reexamination and reconsideration of the application, as amended, are respectfully requested.

2. Compliance with 35 USC 102(b)

The issue is whether Claims 1-3, 5 and 6, and 9-13 are in compliance with 35 USC 102(b) or anticipated by Baumert et al., J. Virol. 72: 3827 (1998). The rule according to MPEP 2131 is that to anticipate a claim, the reference must teach every element of the claim. As evidenced by the attached Comparison Chart, the reference does not teach every element of the claims.

As an initial matter, the rejection for anticipation has been maintained solely with respect to claims 1-3, while claims 5 and 6, and 9-13 have been deemed to be in compliance with 35 USC 102(b).

Referring to the Comparison Chart, this is because the method for isolation of hepatitis C virus (HCV) virus-like particles (VLPs) of Baumert et al. 1998 involves the steps of lysis of cells in a buffer containing a mild non-ionic detergent, either 0.1% NP-40 or 0.5% digitonin, plus the protease inhibitors aprotinin and leupeptin; and pelleting the lysate over a sucrose cushion to form a precipitate.

Beginning with Method 1 (claims 1-3), and still referring to the Comparison Chart, this method involves the steps of lysis of cells in a buffer containing digitonin plus protease inhibitors; and admixing a polyethylene glycol with the lysate to form a precipitate. As indicated by the Comparison Chart, the element of "precipitating with a polyethylene glycol" is missing from Baumert et al. 1998. Contrary to the position by the Patent Office, this element is expressly recited in Claims 1-3, explicitly in step (b) of Claim 1, namely "adding polyethylene glycol to the lysate to form a precipitate". The conclusion is that Baumert et al. 1998 fails to anticipate claims 1-3. Thus claims 1-3 are in compliance with 35 USC 102(b).

Turning to Method 2 (claims 5 and 6), and still referring to the Comparison Chart, this method involves the steps of lysis of cells in a buffer containing digitonin plus protease inhibitors, where the concentration of digitonin is less than or equal to 0.25%; and pelleting the lysate over a sucrose cushion to form a precipitate. As indicated by the Comparison Chart, the element of "the concentration of digitonin being less than or equal to 0.25%" is missing from Baumert et al. 1998. As agreed by the Patent Office, the conclusion is that Baumert et al. 1998 fails to anticipate claims 5 and 6; thus claims 5 and 6 are in compliance with 35 USC 102(b). Compliance with 35 USC 103 is discussed below.

Concluding with Method 3 (claims 9-13), and still referring to the Comparison Chart, this method involves the steps of a hypertonic wash and hypotonic shock of cells; lysis of same in a buffer containing digitonin plus protease inhibitors; and pelleting the lysate over a sucrose cushion to form a precipitate. As indicated by the Comparison Chart, the elements of "hypertonic wash and hypotonic shock" are missing from Baumert et al. 1998. As agreed by the Patent Office, the conclusion is that Baumert et al. 1998 fails to anticipate claims 9-13; thus claims 9-13 are in compliance with 35 USC 102(b). Compliance with 35 USC 103 is discussed below.

Comparison Chart comparing Baumert 1998, Method 1, Method 2, and Method 3

	Baumert 1998, Referring to Materials and Methods - Purification of HCV-like particles on p. 3828 and Results on p. 3831	**Method 1 (Claims 1-3)**, referring to Spec. at Paragraph [0046] and Example 2	**Method 2 (Claims 5-7)**, referring to Spec. at Paragraph [0047] and Example 11	**Method 3 (Claims 9-13)**, referring to Spec. at Paragraph [0048] and Example 18
Hypertonic wash	None	None	None	Hypertonic wash
Hypotonic shock	None	None	None	Hypotonic shock
Lysis	Lysis in a buffer containing a mild non-ionic detergent, either 0.1% NP-40[1] or 0.5% digitonin + Protease inhibitors[2]	Lysis in a buffer containing digitonin + Protease inhibitors	Lysis in a buffer containing ≤ 0.25% digitonin + Protease inhibitors	Lysis in a buffer containing digitonin + Protease inhibitors
Pelleting	Pellet over a sucrose cushion	Precipitate with a polyethylene glycol	Pellet over a sucrose cushion	Pellet over a sucrose cushion

[1] Synonyms: Nonylphenylpolyethylene glycol; Polyethyleneglycol-p-isooctylphenyl ether; Octylphenoxy polyethoxy ethanol; Polyethylene Mono(nonylphenyl)ether Glycols; Polyoxyethylene (9) Nonylphenyl Ether; nonyl phenol ethoxylate; nonylphenyl polyethyleneglycol ether, nonionic; polyethylene glycol 450 nonyl phenyl ether, nonionic surfactant; Tergitol NP-9; polyethylene glycol 450 nonyl phenyl ether; Ethoxylated nonylphenol; alpha(nonylphenyl)-omega-hydroxypoly(oxy-1,2-ethanediyl); polyethylene glycols mono(nonylphenyl)ether; macrogol nonylphenyl ether; nonoxinol; polyoxyethylene(n)-nonylphenyl ether; nonylphenoxypolyethoxyethanol; nonylphenyl polyethylene glycol ether; nonoxynol; Makon; T-DET-N; surfionic n; sterox; arkopal N-090; carsonon N-9; igepal co-630; neutronyx 600; PEG-9 nonyl phenyl ether; protachem 630; rewpol hv-9; polyoxyethylene nonyl phenol; Glycols, polyethylene, mono(nonylphenyl) ether; Nonylphenol polyethylene glycol ether; Nonylphenol, polyoxyethylene ether; Nonylphenoxypoly(ethyleneoxy)ethanol; POE nonylphenol; Polyoxyethylene nonylphenol; POE (10) nonylphenol.

[2] Aprotinin and leupeptin.

3. Compliance With 35 USC 103

The issue is whether claims 5 and 6, and 9-13 are in compliance with 35 USC 103 or unpatentable as being obvious over Baumert et al., J. Virol. 72: 3827 (1998). As it turns out, the prior art actually teaches away from the claimed methods.[1] The rule according to MPEP 2145 is that teaching away is a significant factor to be considered in determining obviousness, where the prior art criticizes, discredits, or otherwise discourages the solution claimed. Under MPEP 2145 X D 3, proceeding contrary to accepted wisdom is evidence of nonobviousness.

According to the patent specification paragraph [0040], the problem in the prior art was that the Baumert et al. 1998 method resulted in low quantities and impure yield. The inventors proceeded contrary to accepted wisdom because, as described in Ghim et al., Virology 190: 548 (1992), attached, previous investigators FAILED to observe human papillomavirus (HPV) virus-like particles (VLPs) AT ALL (p. 552, col. 1, penultimate paragraph) despite (1) immunoprecipitation (p. 550, col. 1), (2) lysis of cells in a buffer containing a mild non-ionic detergent (p. 550, col. 1) and (3) hypertonic shock in glycerol (p. 550, col. 2). In contrast, the present inventors SUCCEEDED in isolating VLPs, although (1) Method 1 is distinguishable from Baumert et al. 1998 by the step of precipitation (Example 2); (2) Method 2 is distinguishable from Baumert et al. 1998 by the step of lysis of cells in a buffer containing a mild non-ionic detergent (Example 11); and (3) Method 3 is distinguishable from Baumert et al. 1998 by the step of hypertonic wash/ hypotonic shock (Example 18). Ghim et al 1992 teaches away from the claimed methods, because it criticizes, discredits, and otherwise discourages the solutions claimed in each of Methods 1, 2, and 3.

For these reasons, the prior art would teach away because a person of ordinary skill in the art, upon reading the references, would be led in a direction divergent from Methods 1, 2, and 3. The conclusion is that the claims are non-obvious over the references. Thus, the claims are in compliance with 35 U.S.C. §103.

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[1] This is not a concession that a prima facie case of obviousness has been established or that any claim limitations are taught or suggested by the prior art reference or references when combined.

4. Rescission and Retraction of Prior Traversal of Restriction Requirement

The claims of the present application were subject to a restriction requirement. In response to the restriction requirement, Applicant provisionally elected with traverse. Applicant hereby rescinds and retracts such traversal.

5. No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, the Applicants are not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. The Applicants reserve the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that the Applicants have made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

Applicant respectfully requests that a timely Notice of Allowance be issued in this case. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 12/14/07

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VIROLOGY 190, 548–552 (1992)

HPV-1 L1 Protein Expressed in cos Cells Displays Conformational Epitopes Found on Intact Virions

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Seven polyclonal and monoclonal antibodies were characterized for their ability to react specifically with either conformational or nonconformational epitopes of the HPV-1 virion. Using these antibodies, it was shown that the HPV-1 L1 protein (when expressed by an SV40 vector in cos cells) displayed conformational epitopes characteristic of intact viral particles. In addition, the L1 capsid protein was translocated normally into cell nuclei, was of appropriate size (57 kDa), and could be isolated in native form by immunoprecipitation techniques. Most importantly, the screening of expressed papillomavirus capsid proteins for reactivity with conformation-dependent antibodies represents a new, general methodology for ensuring that such proteins will be suitable for use in vaccine development or in the serologic detection/typing of human papillomavirus infections. © 1992 Academic Press, Inc.

Papillomavirus infections cause cutaneous warts and mucosal condylomata in a wide variety of vertebrate animals (*1*) and, in humans, are strongly associated with the development of cervical dysplasia and carcinoma (*2*). Each papillomavirus type is highly species-specific and preferentially infects squamous epithelium at a restricted number of anatomic locations. Vegetative viral DNA replication occurs in the nucleus of terminally differentiated keratinocytes where the viral genome becomes encapsidated by the major (L1) and minor (L2) capsid proteins, forming virions 55 nm in diameter. Unfortunately, there are no tissue culture systems which permit sufficient keratinocyte differentiation to propogate papillomaviruses *in vitro* and this limitation has compromised the analysis of the late expression of the L1 and L2 genes as well as the characterization of the host immune response to their gene products.

Due to the etiologic role that human papillomaviruses (HPVs) play in some human malignancies, recent attention has been focused on the development of a recombinant capsid protein vaccine to reduce the incidence of HPV infection and its neoplastic sequelae. The first animal model for a potential vaccine utilized bovine papillomavirus type 1 (BPV-1). The L1 protein of BPV-1 was expressed in bacteria (*3*) and used to immunize cattle against subsequent viral challenge (*4*). However, since the expressed L1 protein apparently lacked native conformation (due to the insoluble, aggregate form of overexpressed fusion proteins in bacteria), it did not induce antibodies which could either recognize or neutralize intact BPV-1 virions (*5, 6*).

The ability of antibodies to neutralize papillomaviruses appears to be related to their ability to react with type-specific, conformational epitopes on the virion surface (*6–9*) and, indeed, previous studies have demonstrated that the predominant antibody response detected against HPV-1 in humans is directed against such conformational epitopes (*10, 11*). In the current study, we characterize a series of antibodies for their reactivity with HPV-1 conformational epitopes and demonstrate that HPV-1 L1 protein synthesized in cos cells expresses these virion conformational epitopes. This expressed protein can, therefore, be used for vaccine development as well as serologic screening techniques.

The initial experiments were designed to characterize a series of polyclonal and monoclonal antibodies for their reactivity with HPV-1 virions which were either in an intact (native conformation) or SDS-denatured (nonconformational) state. It was essential to characterize these antibodies in detail so that they could be used to evaluate the conformational state of expressed HPV-1 L1 protein. A summary of the ELISA experiments and the details for the isolation and purification of the HPV-1 virions are given in Table 1. Briefly, microtiter plate wells were coated with either intact or SDS-disrupted HPV-1 virions as described previously (*12*) and used to screen the indicated antisera or monoclonal antibodies. The two hyperimmune rabbit sera produced against HPV-1 have been described previously (*13*); rabbit (R3) antiserum was generated against disrupted HPV-1 particles and rabbit (R7) antiserum against intact particles. The four monoclonal antibodies that rec-

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TABLE 1

REACTIVITY OF RABBIT POLYCLONAL ANTISERA AND MURINE MONOCLONAL ANTIBODIES WITH INTACT AND DISRUPTED HPV1 VIRIONS[a] AS DETERMINED BY ELISA

Antibody		Immunogen	ELISA value: Intact virions	ELISA value: Disrupted virions
Rabbit	R7	Intact HPV1	1.493	0.002
	R3	Disrupted HPV1	0.918	0.616
Murine	334B6	Intact HPV1	0.438	0.003
	339B6	Intact HPV1	0.520	0.000
	405 D5	Intact HPV1	0.429	0.009
	D5 4G10	Intact HPV1	0.464	0.003
	MAB45[b]	L1 of HPV1	0.512	0.332

[a] HPV-1 virions were extracted from productively infected plantar warts (*20*) and purified by equilibrium centrifugation in a CsCl gradient (*12*). Virions (1.34 g/ml) and empty particles (1.29 g/ml) were collected separately, dialyzed against Tris buffer (20 m*M* Tris, 10 m*M* PMSF, pH 7.5) and stored at −70°. Microtiter plate wells (Immunolon II, Dynatech) were coated with either intact or SDS-disrupted HPV-1 virions as described previously (*12*). The plates were then washed with PBS containing 0.05% Tween 20 (PBST). The microtiter wells were further incubated with PBS containing 1% bovine serum albumin (PBSA) for 1 hr at 37° to prevent nonspecific protein binding. The plates were washed again with PBST and incubated first with either rabbit polyclonal antibodies or murine monoclonal antibodies as primary antibody and subsequently with appropriate alkaline phosphatase-conjugated goat anti-IgG diluted 1:1000 in PBSA (Bio-Rad) for 1 hr at 37°. Following several washes, the microtiter plates were developed with SIGMA 104 phosphatase substrate (Sigma) in diethanolamine buffer (*21*) for 30 min at 37°. Absorbance was measured at 410 nm using a Dynatech Micro-ELISA reader.

[b] MAB45 is an abbreviated designation for MABDW45 (*14*).

ognize conformational epitopes on the surface of HPV-1 particles were kindly provided by Dr. P. Pothier (Bourgogne University, France). Monoclonal antibody (MAB45) defines a linear epitope on the surface of the HPV-1 virion (*14*) and was obtained through the generosity of Dr. D. A. Baker (State University of New York, Stony Brook). The ELISA data indicate that R7 antiserum indeed is specific for conformational epitopes on the surface of the HPV-1 virion since it reacts only with intact HPV-1 virions. This is also true for monoclonal antibodies 334B6, 339B6, 405D5, and D54G10. On the other hand, R3 antiserum and monoclonal MAB45 also react well with SDS-denatured virions, demonstrating their reactivity with linear, nonconformational epitopes (*12*).

To confirm the ELISA results shown in Table 1, we also evaluated the same antibodies for reactivity with disrupted HPV-1 virions as determined by Western blotting (Fig. 1). This figure demonstrates that only antibodies which recognized denatured HPV-1 virions by ELISA (R3 and MAB45) showed significant reactivity with SDS-denatured virion proteins by immunoblotting. However, antibodies shown in Table 1 to recognize only intact virions (R7, 334B6, 339B6, D54G10, and 405D5) exhibited no or little reactivity by immunoblotting analysis. Thus, two independent techniques verify the specifity of the above antibodies for conformational and nonconformational epitopes on the HPV-1 virion.

In an attempt to produce isolated L1 protein which retained critical virion conformational epitopes, we expressed the HPV-1 L1 protein in mammalian cells. The HPV-L1 gene was amplified by PCR and cloned into the pSVL vector as described in Fig. 2 using standard molecular techniques (*15*). The resulting plasmid, pSJ-1, expresses the HPV-1 L1 gene from a strong SV40 late promoter. In addition, the plasmid also contains the SV40 origin of replication and, when transfected into cos cells by calcium phosphate precipitation (*16*), replicates to a high copy number.

Cos cells were first evaluated for L1 protein synthesis by immunoprecipitation techniques using the above antibodies. Forty-eight hours post-transfection, the cos cells were labeled with [^{35}S]methionine (NEN, Express ^{35}S Protein Labeling Mix) for 4 hr, washed with

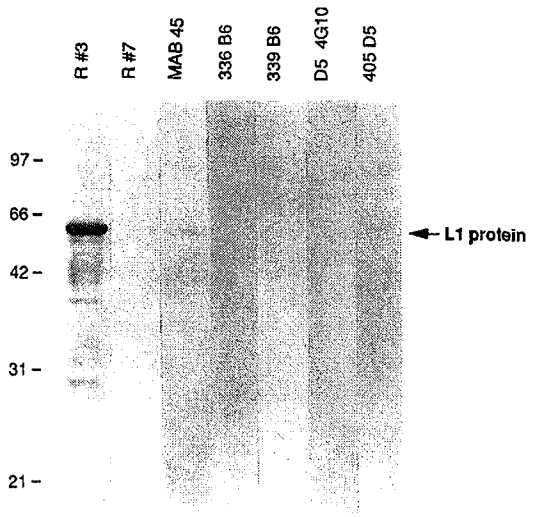


FIG. 1. Reactivity of rabbit polyclonal antisera and mouse monoclonal antibodies with SDS-disrupted HPV-1 as determined by immunoblot analysis. Purified HPV-1 virions were denatured with SDS and their constituent proteins separated by SDS polyacrylamide gel electrophoresis (*12*). The HPV-1 proteins were then transferred electrophoretically to nitrocellulose and reacted with 1:100 dilutions of the rabbit antisera or monoclonal antibodies (ascites fluid). MAB45, which was produced as a hybridoma supernatant, was only diluted 1:10. Primary antibody reactivity was detected using alkaline phosphatase-labeled goat anti-rabbit or anti-mouse IgG (Bio-Rad) at a dilution of 1:1000 in PBSA. Only rabbit antiserum 3 and MAB45, which both recognize denatured HPV-1 virions by ELISA, were found to react significantly with denatured L1 protein (see arrow).

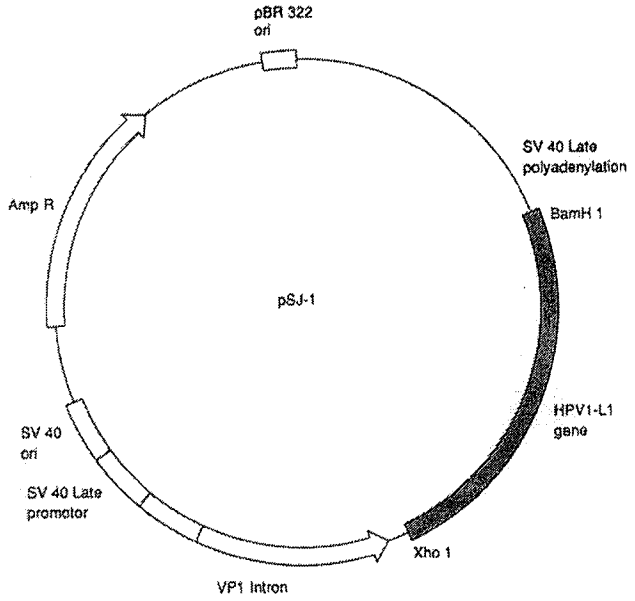


FIG. 2. Construction of SV40 vector, pSJ-1, which expresses the HPV-1 L1 gene. The L1 gene of HPV-1 was amplified from cloned HPV-1 DNA using 5′ and 3′ oligonucleotide primers which contained *Xho*I and *Bam*HI enzyme restriction sites, respectively. The 5′ primer was 5′-TTACATCTCGAGGCCACCATGTATAATGTTTTTC-3′ and the 3′ primer was 5′-GATCCAGGATCCCTAAGCCTTACGCCTGC-3′. Following cleavage with the indicated enzymes, the PCR product was purified and cloned unidirectionally into the pSVL vector (Pharmacia). The resultant plasmid, designated pSJ-1, contained the HPV-1 L1 gene expressed by the SV40 late promoter. The plasmid also contained the SV40 origin of replication (ori) as well as the SV40 VP1 intron and late polyadenylation signals. The entire pSJ-1 L1 gene was sequenced in its entirety and found to be identical to the genomic HPV-1 L1 sequence.

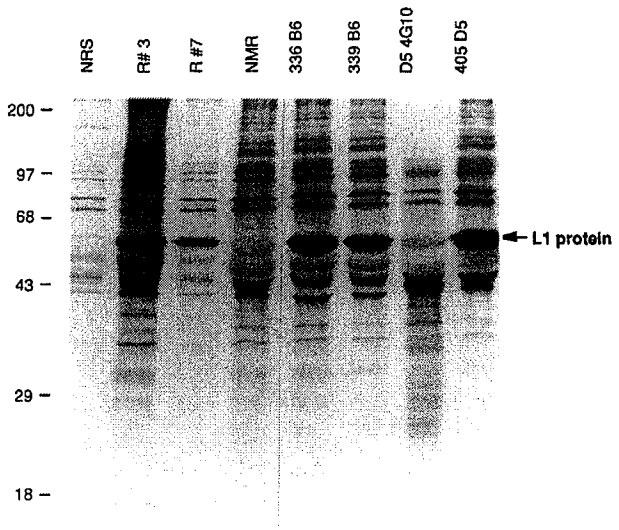
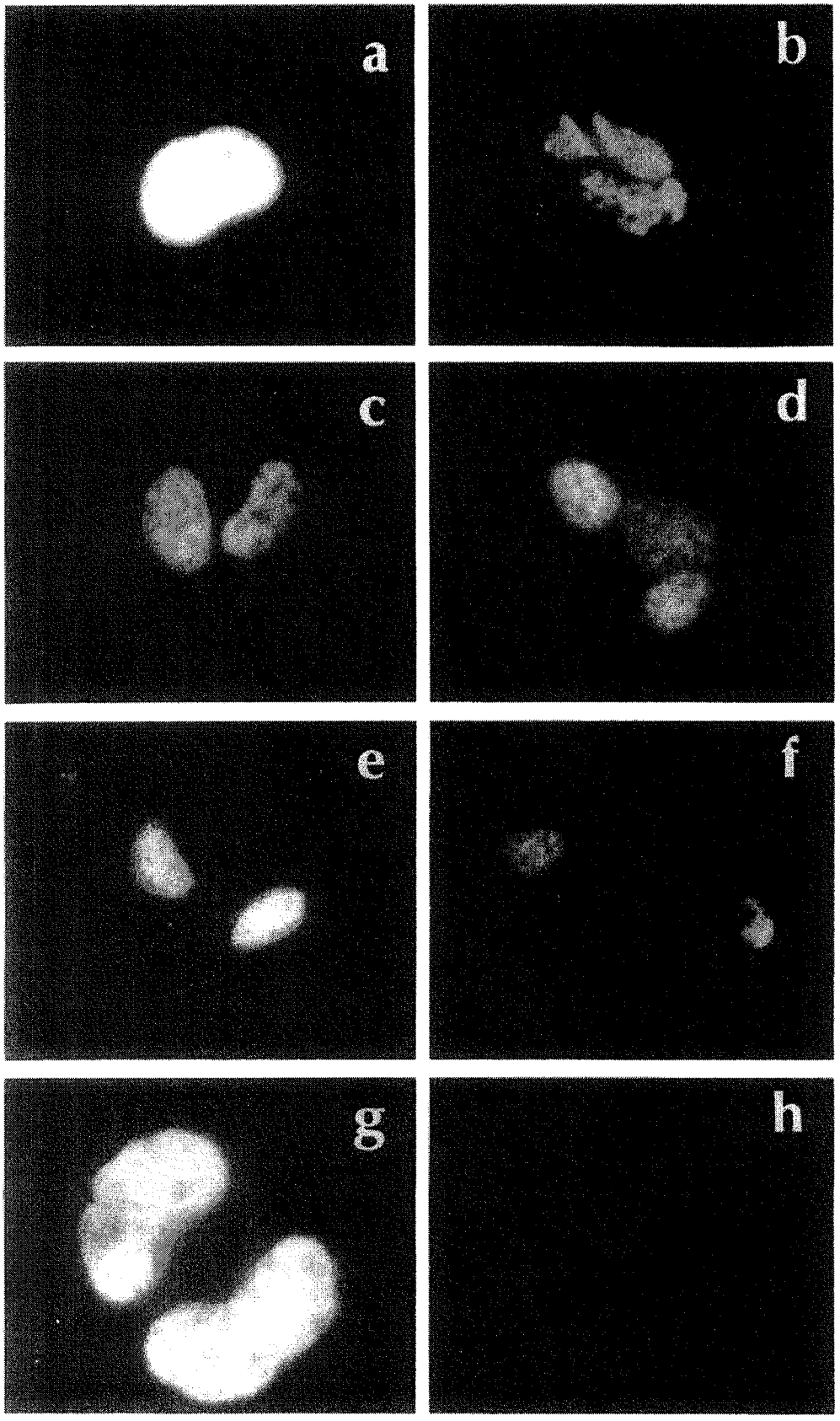


FIG. 3. Immunoprecipitation of HPV-1 L1 protein from cos cells transfected with pSJ-1. Cos cells, grown in 10-cm-diameter plastic plates, were transfected when 80% confluent with 10 μg pSJ-1 plasmid DNA using a calcium phosphate precipitation technique (*16*). Forty-eight hours later, the cells were metabolically labeled with 500 μCi/ml [^{35}S]methionine for 4 hr in 2.5 ml cysteine and methionine-free medium. The cells were then washed with PBS, extracted with RIPA buffer, and immunoprecipitated with the indicated rabbit antisera or mouse monoclonal antibodies. The immunoprecipitated proteins were then analyzed by SDS–gel electrophoresis and autoradiography. All immune polyclonal antisera and monoclonal antibodies were able to immunoprecipitate L1 protein (see arrow). Lanes 1 and 4 show the absence of L1 protein when extracts were precipitated with either nonimmune rabbit serum (lane 1) or with nonimmune murine serum (lane 4).

buffer, and solubilized in RIPA buffer (which contains a mixture of 1% NP-40, DOC, and 0.1% SDS detergents). The cell extracts were then immunoprecipitated with the indicated antibodies and analyzed by SDS–gel electrophoresis as previously described (*17*). The data in Fig. 3 indicate that L1 protein could be efficiently precipitated by conformation-dependent antibodies (such as R7, 334B6, 339B6, D54G10 and 405D5). In addition, the L1 protein could also be immunoprecipitated with antibodies which recognize nonconformational epitopes on the virion surface (R3). These findings indicate that the L1 protein expressed in cos cells displayed conformational epitopes observed previously only on intact virions. It is also obvious that the L1 extraction conditions did not significantly denature the protein. Characteristic of L1 protein isolated directly from virions, the synthesized L1 protein was approximately 57 kDa in size (*18*). The retention of conformational epitopes in RIPA buffer and the ability of conformation-dependent antibodies to react with L1 indicates that the affinity purification of L1 protein from transfected cells will be possible.

Cos cells were also evaluated for L1 protein synthesis by immunofluorescence microscopy (Fig. 4). Cells, plated onto glass coverslips in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, were transfected with 10 μg plasmid DNA, glycerol shocked 48 hr later, washed with phosphate-buffered saline (PBS), and fixed for 5 min in cold ace-

FIG. 4. Immunofluorescent staining of cos cells transfected with pSJ-1. Cos cells grown on glass coverslips were transfected with 10 μg pSJ-1 as described in Fig. 3. After 48 hr, the coverslips were washed with PBS, fixed in cold acetone, and reacted with 1:250 dilutions of rabbit antisera or mouse monoclonal antibodies. The reacted primary antibodies were stained with FITC-labeled goat anti-IgG at the dilution of 1:10 in PBS (Cappel). Nuclei of approximately 5–10% of transfected cos cells were positive by immunofluoresence. The evaluated antibodies were R3 (panel a), R7 (panel b), MAB45 (panel c), 334B6 (panel d), 339B6 (panel e), D54G10 (panel f), and 405D5 (panel g). All antisera were nonreactive with cos cells transfected with the parent pSVL vector lacking the HPV-1 L1 gene, including R3 (panel h).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tone. The cells were then reacted with appropriate dilutions of primary antibody followed by fluorescein-conjugated goat anti-rabbit or goat anti-mouse IgG. Incubations with primary and secondary antibodies were performed at room temperature for 1 hr. Subsequent to a final PBS wash, the coverslips were mounted in Elvanol and viewed with an Olympus fluorescent microscope. The presence of L1 protein in cell nuclei was clearly discernable in 5–10% of transfected cells 48 hr post-transfection, independent of whether the primary antibody reacted with conformational and/or nonconformational epitopes. All of the antibodies which were capable of immunoprecipitating L1 were also successful by immunofluorescence. As mentioned previously, antibodies produced against disrupted virions recognize both internal and external virion linear epitopes and therefore are capable of reacting with intact particles (e.g., R3). However, such antibodies do not recognize conformational epitopes and are not neutralizing (*6*). Thus, the staining pattern obtained with rabbit antisera to native (R7) or denatured (R3) HPV-1 virions was indistinguishable. These results, therefore, demonstrate unequivocally that the L1 protein synthesized in the cos cells was of a conformation similar to that found in intact virions. In addition, the protein clearly translocated to the nucleus in a normal fashion (*19*).

The above findings suggest that the HPV-1 major capsid protein, when expressed in the absence of other viral proteins, can precisely reproduce/mimic the antigenicity of intact viral particles. While we cannot be certain that no assembled viral particles are present in the transfected cos cells, we have been unsuccessful in visualizing such structures by electron microscopic examination of either transfected cells or of immunoprecipitates containing L1 protein (data not shown). Apparently it is not essential to have viral particle formation in order to reproduce the characteristic, viral conformational epitopes.

Since the neutralization sites present on papillomavirus virions consist predominantly of conformational epitopes, it is inferred in our studies that the L1 protein synthesized in cos cells might serve successfully as a vaccine or for the serologic detection and typing of papillomavirus infections. Due to the similarities among the papillomaviruses with respect to genetic organization, virion structure, and amino acid sequence of their capsid proteins, it is also likely that our findings with HPV-1 L1 will have direct applicability to the study of other HPVs such as HPV-16 and HPV-18 which have important contributory roles to the development of cervical carcinoma.

ACKNOWLEDGMENTS

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